Synopsis

The glycoprotein hormone family comprising of Luteinizing Hormone (LH), Chorionic Gonadotropin (hCG), Follicle Stimulating Hormone (FSH) and Thyroid Stimulating Hormone (TSH) plays important role in reproduction and overall physiology of the organism These hormones are heterodimeric molecules consisting of an identical α subunit non-covalently associated with the hormone-specific β subunit Both subunits of all these hormones are *N*-glycosylated In addition, hCGβ subunit also has four O-linked oligosaccharides located at the C-terminus of the polypeptide(1) The α and β subunits of all these hormones contain five and six disulfide bonds respectively and the crystal structures of hCG and hFSH indicate that both subunits of the hormones belong to the cystine knot family of proteins(2-4) Although the β subunits are hormone specific, there are distinct similarities in these subunits with the 12 cysteines conserved in all these subunits (1). These hormones, because of their unique structural features have proved to be important models for structure–function relationship studies of complex dimeric glycoproteins. Folding of subunits during biosynthesis, role of glycosylation in folding pathways and *in vitro* and *in vivo* bioactivity of the hormone, as well as, identification of domains important for subunit association, receptor binding and subsequent signal transduction have been topics of active investigations

The receptors of these hormones belong to the family of G-protein coupled receptors (GPCR) and have unique hormone specific exodomain not present in other members of the GPCR family and characteristic seven transmembrane domains followed by a C terminal domain(5) Primary structure analysis of Glycoprotein hormone receptors family revealed sequence conservation, maximum homology being observed in the transmembrane domain (TMD)(6) Significant homologies could be observed in the hormone specific extracellular domains (ECD) also (7) Despite these homologies, the receptors exhibit exquisite specificity with very low cross reactivity with other members of the hormone family (8)

Elucidation of the molecular details of the contacts between the hormone and the receptors has not been achieved so far. Various approaches have been employed to delineate the residues or domains of both hormone and receptors involved in interaction These include testing of chimeras or mutants of hormones or receptors for changes in activity (9-12), chemical modifications(13) and competition with peptides from either hormones (14) or receptors(15). Polyclonal and monoclonal antibodies against glycoprotein hormones and various fragments of their receptors have been used to determine the role of different domains of both in binding and response (6, 16, 17) However, till date there is no consensus on the specific mechanisms by which the glycoprotein hormone docks onto its receptor It was proposed that the initial contact between the hormone and the receptor occurs through high affinity binding of the hormone specific β subunit to the Leucine rich regions of the ECD that results in conformational changes in both hormone, as well as, the receptor and brings hormone/ECD complex closer to the TMD of the receptor. The secondary, relatively lower affinity interactions between the hormone and the receptor then take place through common α subunit and exoloops of TMD of the receptor resulting in signal generation (18, 19). Recently a different kind of model has been proposed which suggests that the hormone does not make any direct contacts with the TMD of the receptor. The signal is

transduced by the change in contacts between ECD and TMD brought about by hormone's interaction with ECD(8, 20)

The present study was initiated with an overall objective of understanding the molecular details of the hormone receptor interactions of this family, particularly hCG-LH receptor interactions Two different approaches were employed for this purpose; the first, direct approach being structure elucidation of the members of the glycoprotein hormone family while the second approach uses antibodies against hCG as tools to probe into hormone-receptor interactions The results obtained using these two approaches have been consolidated in the present thesis and are organized as follows

Chapter 1 is an extensive review of the literature and it builds background for the present work while the exact aim and scope of the present work have been defined in Chapter 2 Chapter 3 describes cloning, expression and purification of recombinant glycoprotein hormones hLH, hCG and single chain derivative of hCG The Chapter 4 gives details of the molecular aspects of hCG-LH receptor interaction dissected using hCG monoclonal antibodies (MAbs) Chapter 5 discusses implications of the observations made in the present study and states the future directions envisaged

There are a number of endocrinopathies associated with abnormal levels of glycoprotein hormones and treatments of such disorders often demand large quantities of either agonists or antagonists of the hormones The structure-function relationship studies should help in identifying domains/residues important for subunit interaction, receptor binding, and signal transduction, which would also allow engineering of agonists and antagonists of hormone action. However, structure determination of the glycoprotein hormone family using X-ray crystallography has proved to be a difficult task and it is believed that the heterogeneity in glycosylation is the primary reason for this low success rate in the process of crystallization The first crystal structure of hCG was that of completely deglycosylated hCG but such a molecule displays antagonistic behavior(2, 3) Use of NMR spectroscopy, the alternate method commonly used for structure determination is often limited by the availability of large quantities of biologically active hormones free of any contaminants Large quantities of LH, hCG and FSH are also required for treatment of infertile patients suffering from gonadotropin deficiency The first goal of the present study was thus to produce and purify biologically active recombinant hCG and hLH. Owing to the inherent features of glycoprotein hormones and their potential therapeutic applications, the recombinant expression of these hormones is an important goal from both basic research, as well as, commercial point of view. Considering the above mentioned features it is clear that the expression system used for the hyperexpression of these glycoprotein hormones should also serve as a model system for investigating structure–function relationships and folding of subunits during biosynthesis, in addition to providing sufficient quantities of the hormones for clinical applications. It has been demonstrated that *N*-linked glycosylation during biosynthesis facilitates protein folding and conformational maturation of glycoprotein hormone subunits into an assembly-competent, biologically active form (21). Therefore, the ideal recombinant expression system should also be able to glycosylate the protein during biosynthesis The *Pichia pastoris* yeast expression system was chosen for hyperexpression of glycoprotein hormones as it blends the advantages of both bacterial and mammalian expression systems. Earlier, expression of biologically active hCG and the subunits of hCG and bovine FSH using *Pichia pastoris* expression system has been

reported from the laboratory (22, 23) Chapter 3 (section 3 3 1) of the thesis describes hyperexpression of hLH. The expression of these heterologous proteins was scaled up using fermentation procedures to fulfill the requirements of large quantities of hormones for various applications Purification of Pichia expressed hormones turned out to be a complex task as large quantity of the hormone was secreted out in the fermentation medium (10litre volume) that was of high ionic strength Of several different strategies attempted for concentration and partial purification of recombinant hCG, hydrophobic interaction chromatography (HIC) using Phenyl Sepharose matrix emerged as the most efficient technique as a first step of purification. Subsequently, cation exchange chromatography using SP- Sepharose matrix yielded completely purified biologically active recombinant hCG (section 3 3 2) The preliminary data also suggested that Pichia cells express a biologically active form of hCG which appeared to be less glycosylated and of lower molecular weight Using the same protocol purification of hLH, as well as, single chain derivative of hCG, phCG$\alpha\beta$ was achieved (section 3 3 3) These recombinant proteins were characterized extensively using various biochemical, as well as, immunological criteria and were shown to be similar to their natural counterparts with respect to their ability to bind LH receptor and to transduce signal as judged by radioreceptorassays and *in vitro* bioassays respectively The hydrophobic interaction chromatography proved an important starting point for purification of all the other members of the glycoprotein hormone family expressed using *Pichia pastoris* expression system

With the availability of purified, biologically active recombinant hCG in large quantities it was now possible to make attempts towards structure elucidation using NMR spectroscopy The structure determination of such complex proteins by NMR spectroscopy is made relatively easier by labeling the proteins with magnetically more active, stable isotopes of carbon and nitrogen, ^{13}C and ^{15}N respectively however the cost is often prohibitively high The *Pichia pastoris* expression system offers simple means of labeling the proteins as the cells can be grown on simple salts of carbon and nitrogen such as ^{13}C labeled methanol, ^{15}N labeled ammonium chloride or ammonium sulphate. The Chapter 3 also gives a brief account of the preliminary attempts made to label the recombinant hCG with ^{15}N and the structural studies carried out with the carbohydrate moieties of the recombinant hCG using solution NMR spectroscopy. This work was carried out in collaboration with the laboratory of Prof. J.P Kamerling of the University of Utrecht, Netherlands and the efforts are currently underway to elucidate the complete structure of the *Pichia* expressed hCG.

The common feature of receptors and antibodies against the ligand is that both display very specific, high affinity binding towards the ligand. Hence, it is logical to speculate that the antigen binding regions of the antibodies that inhibit hormone binding and/or response, exhibit homology with distinct domains of the receptor. By identifying the epitopes recognized by such antibodies, it should be possible to predict contact points between the hormone and the receptor. In the present study, this hypothesis has been tested using monoclonal antibodies (MAbs) against hCG recognizing different epitopes in the hormone molecule and having different effects on hormone binding and response (Chapter 4). These MAbs were classified as α subunit specific, β subunit specific or heterodimer specific depending on their abilities to bind either subunit in addition to the hormone itself. Interestingly, it was observed that the hCGβ subunit specific MAbs, as

well as, heterodimer specific MAbs inhibited hCG receptor binding and hence the response generated by hCG, while the hCGα subunit specific MAbs inhibited only response to the hormone without interfering in binding (Section 4.3 1) To dissect out these interactions further the epitopes recognized by these antibodies on hCG molecule were determined (Section 4 3 2), single chain fragment variable (ScFv) were generated from each of these antibodies and it was shown that these ScFv retain the functionality of the original antibody (Section 4 3 3) Further, the amino acid sequence of each antibody was determined (Section 4 3 4) and finally shown that the antigen binding domains of antibodies show homology to the distinct regions of the LH receptor on sequence alignments between the two using three different programs (4 3 5) The hCGβ subunit specific MAb 52/28' displayed distinct homology with the ECD of LH receptor while the α subunit specific MAb C10 showed regions homologous to TMD of the receptor and the heterodimer specific MAb E12 was found to be similar to the hinge region of the receptor This clearly indicates that the β subunit of hCG is in close contact with ECD of the receptor while the α subunit makes contacts with the TMD of receptor. The present study thus supports the existing model of hormone receptor interactions, which states that the hormone first binds to the exodomain of the receptor mainly through its β subunit while the integrity of the α subunit is critical for signaling (24, 25) Also, the observations made in the present study exhibit an interesting possibility of antigen antibody complexes being used as surrogate models for gaining insights into hormone receptor complex

Further, it has been reported that hCG has immunocontraceptive potential(26) Active and passive immunization studies with hCG in primates and humans have demonstrated the possibility of controlling fertility by the antibodies capable of neutralizing hCG. This forms the basis for female contraceptive vaccine that has undergone Phase II clinical trials in India The MAb E12 characterized in the present study displayed highly specific binding to heterodimeric hCG exclusively without showing any cross reactivity with hLH (Section 4 3.1) The epitope mapping analysis revealed that this antibody recognizes a unique discontinuous epitope present only in the heterodimeric hCG and is distinct from the unique C-terminal extension of hCGβ absent in hLHβ (Section 4 3 2) The MAb, IgG or its recombinant single chain fragment variable (ScFv), inhibited response to hCG, but not to hLH (4 3 3). Thus, the epitope recognized by this MAb is an ideal candidate antigen for immunocontraception The MAb E12 can also be used for passive immunization in case of emergency contraception. Another potential application of hCG specific antibodies is in homing and the treatment of tumors that secrete hCGβ subunit The hCGβ subunit specific MAbs used in the present study 52/12 and 52/28' that inhibited hCG receptor binding as well as response generated by hCG can be used in treating such tumors The functional ScFvs generated from these MAbs in the present study can be made use of on humanization. Thus, the present study has yielded some important molecules for therapeutic applications besides providing a new platform for structure-function relationship studies of the complex glycoprotein hormones.

References:

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